THE EFFECT OF ADDITIVE AND DRY MATTER CONTENT ON SILAGE PROTEIN DEGRADABILITY AND BIOGENIC AMINE CONTENT

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ABSTRACT. *Legumes are widely used ensiling material as they are rich in protein. Ensiling of legumes arises several problems due to their low content of sugars, high buffering capacity and high moisture content. Attention should be paid to silage protein degradability. Objective of the study was to explain the effect of additives and wilting on the fermentation quality and nutritive value of red clover-timothy silage, protein degradability and content of amines included.*

Test silages from fresh material, either unwilted or wilted for 24 hours, were conserved into 3-litre jars and opened for analysis in 90 days. Biological (L. plantarum +L. fermentum) and chemical (AIV 2000) additives were used for treatment. Silage protein degradability was studied by using in sacco *method with ruminally fistulated cows.*

As the buffering capacity of red clover-timothy mixture (50:50) is low (27.5), increasing up to 39.6 at wilting, treatment with additive is necessary to improve fermentation. The use of biological or chemical additives decreased silage dry matter losses by 1.9 to 3.7 times, significantly improving the quality of fermentation – content of butyric acid was 47 g/kg DM for test silage and 1–2 g/kg DM or 0 for silage without additive.

In vitro *organic matter digestibility for the silage with chemical additive increased by 4%, compared to that for the test silage (P<0.0001).*

Ruminal degradability of silage nitrogen was approximately 90%.

Protein solubility and ruminal degradability were lower for silage with chemical additive. Ruminal degradability of silage protein after 8 h was 77.2% for the test silage, 76% for the silage with biological additive and 68% for the silage with chemical additive.

All biogenic amines under investigation were present in low dry matter (140g/kg) silages, prepared without an additive. The content of histamine was the highest (5.24 g/kg DM), followed by putrescine (0.86 g/kg DM). Wilting and treatment with additive significantly decreased the formation of biogenic amines in silages.

Keywords: *red clover, silage, protein, degradability, amines.*

Introduction

Legumes are essential feed cultivars in dairy cattle diets. As there is a need to reduce the use of inorganic nitrogen fertilisers in agriculture, the role of legumes among feed cultivars is becoming more important. Legumes fix atmospheric nitrogen through their symbiotic association with *Rhizobium* bacteria. Due to high nitrogen content, the use of legumes as feed cultivars is particularly of great interest.

High protein content of legumes can arise several problems related to feeding and nitrogen emission. Research results reveal that legume protein is intensively degradable during drying (Makoni *et al.*,. 1993, Givens, Rulquin, 2002), ensiling (Makoni *et al*., 1993; Kohn, Allen, 1995, Jurgens, 1997) or ruminal fermentation (Dewhurst *et al.*, 2003). High protein degradability of legumes decreases the efficiency of protein utilisation as a protein source, which may increase milk production but decrease its protein content (Hojman, 2004). High amount of protein in diet arises fertility problems (Butler, 1998; Rajala-Schultz *et al*., 2000), makes milk renneting characteristics worse (Castillo, 1999), increases the occurrence of metabolical disorders (Zhu *et al*., 2000) etc.

Little attention has been paid to the content of different protein end-products in silage and their effect on the animal organism. The given research tried to explain the effect of silage additive on the content of biogenic amines, besides other indicators of silage quality and protein hydrolysis.

The most common biogenic amines in silage are putrescine, cadeverine, histamine and tyramine. It has been well approved (Harrison, 1994), that biogenic amines have negative impact on dry matter intake as they inhibit ruminal contractions, reduce dry matter digestibility and the passage rate of feed particles in the digestive tract (Phuntsok *et al*., 1998).

Putrescine, decreasing mainly cows' milk production and dry matter intake, is a factor causing ketosis either alone or with other amines (Lingaas, Tveit, 1992). The fact that histamine introduces laminitis was

demonstrated already in 1963 by Nelsson who injected it under the dairy cows' skin. Some years later Takahashi and Young (1981) got the same results, providing supplemental histamine to the cows fed diets with high starch content.

Due to the increase of cows' production and the use of concentrated dairy cattle diets, the incidences of laminitis have considerably increased (Stone, 2004). The formation of biogenic amines takes place in the organism during catabolism (Zilmer *et al*., 1999), yet in higher amounts they are formed with starch-rich rations during ruminal microbial hydrolysis (Phuntsok *et al*., 1998). If the content of amines in silage is considerable, the risk of incidences increases as well.

Material and methods

The mixture (50:50) of red clover – *Trifolium pratense* L., variety Jõgeva 433 – and timothy – *Phleum pratense* L., variety Tika – was studied. The fresh material was cut at the height of 5 cm. One part of it was ensiled immediately, the rest after 24 hours of wilting. Silage material was chopped into 2 cm pieces. Carefully mixing, additive was sprayed to the silage material. Silages were conserved into 3-litre jars.

All test silages were made with three repetitions. Chemical additive (AIV 2000) and biological inoculant (*L. plantarum* + *L. fermentum*), prepared in the Institute of Microbiology of Tartu University, were used. Both additives were added 5 l per ton of silage. The concentration of lactic acid bacteria in biological inoculant was 8×10^9 cfu/g.

The jars of test silages were opened in 90 days. Dry matter loss was calculated as the difference between dry matter concentration before and after ensiling. The content of volatile fatty acids (VFA), ethanol, pH and ammonia nitrogen (NH_3–N) was determined in water solution. The value of pH was measured by using a pH-meter (MP 120 Mettler Toledo); NH_3-N was determined by Kjeldec Auto 1030 analyser (Foss Tecator); the contents of ethanol, lactic acid and VFA were determined chromatographically (Perkin Elmer 900) by using columns packed with 80/120 Carbopack B-DA/4% carbowax 20 M (Faithfull, 2002).

Samples were dried for 20 hours at 60 °C, chopped into 1mm-diameter-particles and analysed for the content of dry matter, crude protein, crude ash and crude fibre (AOAC, 1990). To determine crude ash concentration, samples were reduced to ashes in furnacle at 550 ºC for 6 hours. Crude protein was analysed by Kjeldahl method with Kjeldec Auto 1030 analyser (FOSS Tecator) and crude fibre by Fibretec system. Additionally, the samples were analysed for *in vitro* organic matter digestibility (IVOMD), neutral detergent fibre (NDF) and acid detergent fibre (ADF), using ANKOM technology and equipment (ANKOM Technology, Fairport, NY USA) – incubator DAISY II and fibre analyser ANKOM 200 (Van Soest *et al*., 1991).

For determination of degradability, the samples were freezed at –20 °C and ground; 4 g of silage dry matter was weighed into bags made from polyester cloth with the pore size of 28 µm. Soluble fraction was determined by washing the bags in a washing machine set to a cold cycle. For determination of readily soluble fraction, the samples were incubated in the rumens of fistulated cows for 8 hours; for potentially degradable fraction the incubation time was 64 hours. Ruminal protein degradability of silage was calculated by nutrient losses from the bags during the incubation period (Varvikko, Vanhatalo, 1992).

For determination of biogenic amines, 10 g of silage sample (freezed at –20 ºC and ground) was added to 20 ml methanol/water solution and mixed for 2 minutes. The sample was incubated for 45 minutes at 45 ºC and cooled to 30 ºC. Filtrate was separated by centrifuging. The obtained filtrate was derivatizied, centrifuged and stored at –20 °C in gas chromatograph (GC) viales (Yen, Hsieh, 1991). GC analysis was carried out using HP 6890 Series GC System with capillary column HP-5.

Fermentability coefficient (FC) was calculated according to Pahlow and Weissbach (1999):

$$FC = DM\ [\%] + 8\ WSC/BC^{-1},$$

where DM – dry matter content %, WSC – water soluble carbohydrates g/kg dry matter, BC – buffering capacity.

Sugars were determined by Bertran method (Thomas, 1977) and buffering capacity by the formula of Pahlow and Weissbach (1999).

Statistical analysis

Data were analysed by using GLM procedure of SAS. The effects of treatment and additive were tested by means of orthogonal contrasts. For analysing the traits containing zero values, ranks of values were used; other traits were transformed to their logarithmic values.

The contrasts were calculated from the following model:

$$Y_{ijk} = \mu + T_i + K_j + E_{ijk};$$

where Y_{ikj} – trait; μ – mean; T_i – treatment; K_j – effect of additive; E_{ijk} – random error.

Results and discussion

Chemical composition and dry matter losses of silage

Material used in the experiment was cut at the heading stage. Dry matter of the fresh material at harvest was 185 g/kg and the content of crude protein, crude fibre and NDF in dry matter was 147 g/kg, 201 g/kg and 432 g/kg, respectively (Table 1). During drying, the content of DM and CP increased, but due to intracellular respiration the content of sugars declined by 7.9%. Ensilability of grasses can be characterised by their fermentability coefficient which considers the content of dry matter and carbohydrates, also buffering capacity of the fresh material. Due to quite low level of rapidly digestible carbohydrates and high level of protein, legumes have a high buffering capacity, resulting in poorly fermented silage (McDonald *et al*., 1991, Wilkinson, 2005). By the opinion of Pahlow *et al.* (2002), silage material has good ensiling properties if the fermentation coefficient is higher than 45. In this study it was considerably lower even after wilting the material.

As microorganisms use organic matter – readily fermentable carbohydrates – for their life activity during fermentation (McDonald *et al*., 1991), the content of nitrogen-free extractives as well as dry matter content in silage was reduced (Table 2), as compared to the fresh material. Nutrient losses were relatively high, ranging to 24.1% for the test silage, prepared from unwilted fresh material with high moisture content. Dry matter losses of unwilted fresh material were lower: 17.4% for the test silage. By the data of Pettersson (1988), dry matter losses of unwilted silage (0.8 to 71.1 %, average 19.4%) were higher than these of wilted silage (13.1 to 13.4%). The tendency that dry matter losses decrease with wilting and additive application (Wilkinson, 2005) was revealed by our investigation as well. Nutrient losses were lower for unwilted material, treated with an additive ($P<0.0001$) (Table 2).

Table 1. Chemical composition, buffering capacity and fermentation coefficient of fresh red clover-timothy mixture before ensiling

Item	Fresh material	Wilted material
Dry matter, g/kg	185	310
In dry matter, g/kg		
crude protein	147	161
crude ash	82	89
crude fibre	201	203
NDF	432	440
ADF	245	245
N-free extractives	537	515
sugars	101	93
buffering capacity*	90	86
Fermentation coefficient	27.5	39.6

* lactic acid (g), needed for titration of 100 g dry matter

Table 2. Effect of additive on silage chemical composition and dry matter losses during ensiling (g/kg DM)

Treatment / silage additive	Dry matter	Crude protein	Crude fibre	N-free extractives	Dry matter losses, %
Unwilted					
test	140	153	274	421	24.1
biological	171	161	220	481	7.7
chemical	162	172	222	471	12.4
Wilted for 24 hours					
test	256	178	209	468	17.4
biological	274	172	192	498	11.6
chemical	295	172	185	506	4.7
	Significant difference, P				
Unwilted					
test *vs*. biological	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
test *vs*. chemical	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
biological *vs*. chemical	0.0005	<0.0001	0.5771	0.0056	0.0005
Wilted for 24 hours					
test *vs*. biological	<0.0001	0.0062	<0.0001	<0.0001	0.0014
test *vs*. chemical	<0.0001	0.0027	<0.0001	<0.0001	<0.0001
biological *vs*. chemical	<0.0001	0.8847	0.0196	0.0194	0.0417

Effect of silage additive and wilting on silage quality

Without an additive, ensilability of both wilted and unwilted silage material was low, as indicated by the characteristics of silage fermentation quality: high pH and butyric acid content, and low lactic acid content in test silages (Table 3). For well-fermented and stable silage, the value of pH is 4.2 or lower with 200 g DM/kg and below 4.75 with 400 g DM/kg (Weissbach, 2003); dry matter content of lactic acid is over 35 g/kg, acidic acid content below 20 g/kg, and butyric acid is not present (Moisio, Heikonen, 1992; Wilkinson, 2005). Ensilability of red clover-timothy mixture was significantly improved by treatment with either biological or chemical additive.

Table 3. Effect of additive on silage fermentation quality and organic matter digestibility

Treatment / additive	pH	Lactic acid, g/kg DM	Butyric acid, g/kg DM	Acetic acid, g/kg KA-s	OMD, %
Unwilted					
test	5.9	14	47	8	66
biological	4.0	43	1	7	76
chemical	4.5	39	2	9	73
Wilted for 24 hours					
test	5.0	74	24	8	74
biological	4.2	110	0	9	77
chemical	4.8	44	0	10	78
	Significant difference, P				
Unwilted					
test *vs*. biological	<0.0001	0.0001	<0.0001	0.1798	<0.0001
test *vs*. chemical	<0.0001	0.0003	<0.0001	0.5620	<0.0001
biological *vs*. chemical	<0.0001	0.7616	<0.0001	0.0605	0.0010
Wilted for 24 hours					
test *vs*. biological	<0.0001	0.1002	<0.0001	0.6379	0.0014
test *vs*. chemical	0.0015	0.0729	<0.0001	0.1229	<0.0001
biological *vs*. chemical	<0.0001	0.0015		0.2727	0.0417

Chemical additive increased the digestibility of organic matter (P<0.0001) by 7% for unwilted silage and by 4% for wilted silage. However, unwilted silage without additive had signs of spoilage (pH 5.9 etc.), probably resulting in digestibility decrease (Muck, Pitt, 1993). Digestibility of the silage treated with chemical additive AIV, has been by 3.8% higher by the data presented by Jatkauskas and Vrotniakiene (1999), and by 3.1% higher by the study of Kaldmäe *et al*. (2001).

Average ruminal degradability of feed protein is 60 to 80%; the degradability of non-protein nitrogen compounds is higher (Kaufmann, 1979). Ruminal degradability of silage nitrogen was approximately 90% as indicated by fraction B3 (Table 4). Wilting of silage material had no effect neither on protein degradability nor degradability rate (Table 4).

Protein solubility was the lowest for the silage with chemical additive – 64.2% (P<0.001) for unwilted silage and 60.0% (P<0.0001) for wilted silage. Chemical additive also decreased the intensity of protein degradability as indicated by degradability of fraction B2, which was lower by 0.2%. The effect of additive on the decrease of silage nitrogen degradability and kinetics has been shown by Kaldmäe *et al.* (2004), and Flores *et al*. (1999), who indicated that silage nitrogen degradability of ryegrass reduced from 81.22 to 79.31%. Hristov and Sandev (1998) concluded that protein solubility of lucerne silage, prepared with chemical additive, decreased by 9.5%.

The proportion of ammonia nitrogen in total nitrogen characterises the rate of protein degradation. The higher the content of ammonia nitrogen in silage is, the higher is the proportion of non-protein nitrogen compounds in total nitrogen and the higher is the amount of soluble nitrogen. Our data revealed that the content of NH_3-N in test silages was 18.7% and 6.3% – considerably higher as that in silages treated with additive. Protein solubility of test silages also tended to be higher as presented in Table 4.

Table 4. Effect of silage additive on protein hydrolysis

Treatment / additive	Total N, g/kg	NH_3-N total N, %	Soluble fraction, % total N B1	Rapidly degradable fraction, % total N B2	Potentially degradable fraction, % total N B3
Unwilted					
test	24.5	18.7	67.2	77.1	89.6
biological	25.8	1.1	65.5	79.8	91.1
chemical	27.5	5.8	64.2	76.1	94.3
Wilted for 24 hours					
test	28.5	6.3	74.7	77.2	92.6
biological	27.5	1.6	65.2	76.0	92.6
chemical	27.5	3.9	60.0	68.0	91.8
	Significant difference, P				
Unwilted					
test *vs*. biological	<0.0001	<0.0001	0.3150	0.0210	0.0025
test *vs*. chemical	<0.0001	<0.0001	0.0003	0.3677	<0.0001
biologigal *vs*. chemical	<0.0001	<0.0001	0.0928	0.0018	<0.0001
Wilted for 24 hours					
test *vs*. biological	0.0062	<0.0001	<0.0001	0.2852	0.9491
test *vs*. chemical	0.0027	0.0034	<0.0001	<0.0001	0.1146
biologigal *vs*. chemical	0.8847	<0.0001	<0.0001	<0.0001	0.1011

Effect of additives on the content of biogenic amines in silage

In our study, considerable amounts of biogenic amines were found only in low dry matter silages with additives (Table 5). The amount of biogenic amines was very low or they were not present in wilted silages which dry matter content of fresh material was 310 g/kg. All biogenic amines under investigation were present in low dry matter silages without additives. Histamine had the highest and purtrescine somewhat lower concentration: 5.24 g/kg and 0.86 g/kg, respectively. Additive totally inhibited the formation of putrescine, histamine and tyramine, and also reduced the formation of cadeverine approximately 100-fold.

In literature not many data can be found about the presence of biogenic amines in silage. Norwegian researchers Krizsan and Randby (2005) have studied the effect of fermentation quality on silage intake. They ensiled fresh material with low dry matter content (166 to 237 g/kg), using various technologies and additives, and besides other fermentation characteristics, they determined the content of biogenic amines. The content of putrescine, cadaverine, histamine and tyramine was 0.17 to 3.73 g/kg; 1.22 to 5.41 g/kg; 0 to 1.43 g/kg and 0.29 to 2.68 g/kg, respectively. Comparing the results of our investigation to these of Norwegian researchers, it can be seen, that histamine content of our unwilted test silage was somewhat higher. However, the data do not reveal species composition, technology used and effect of additive on biogenic amines. All biogenic amines served as factors significantly decreasing intake.

Table 5. Effect of additive on the content of biogenic amines in silage (g/kg DM)

Treatment / additive	Putrescine	Cadeverine	Histamine	Tyramine
Unwilted				
test	0.86	2.32	5.24	2.00
biological	0	0.03	0	0
chemical	0	0.03	0	0
Wilted for 24 hours				
test	traces	0.05	0	0.08
biological	0	0	0	0.01
chemical	0	0.03	0	0

Not many investigations have been made on the effect of biogenic amines on ruminant metabolism. As feeding poorly fermented silage to dairy cows increases the incidences of metabolical disorders and decreases milk quality, more attention should be paid to the problems associated with the presence of biogenic amines in milk.

Summary

As the buffering capacity of red clover-timothy mixture (50:50) is low (27.5), increasing up to 39.6 at wilting, treatment with additive is necessary to improve fermentation. The use of biological or chemical additives decreased silage dry matter losses by 1.9 to 3.7 times, significantly improving the quality of fermentation – content of butyric acid was 47 g/kg DM for test silage and 1–2 g/kg DM or 0 for silage without additive.

In vitro organic matter digestibility for the silage with chemical additive increased by 4%, compared to that for the test silage ($P<0.0001$).

Ruminal degradability of silage nitrogen was approximately 90%.

Protein solubility and ruminal degradability were lower for silage with chemical additive. Ruminal degradability of silage protein after 8 h was 77.2% for the test silage, 76% for the silage with biological additive and 68% for the silage with chemical additive.

All biogenic amines under investigation were present in low dry matter (140g/kg) silages, prepared without an additive. The content of histamine was the highest (5.24 g/kg DM), followed by putrescine (0.86 g/kg DM). Wilting and treatment with additive significantly decreased the formation of biogenic amines in silages.

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